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6. AUTHOR(S)

John G. Duman

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Department of Biological Sciences
University of Notre Dame
Notre Dame, IN 46556

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

AFOSR/NL
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13. ABSTRACT *(Maximum 200 Words)*

Antifreeze proteins (AFPs) from overwintering larvae of the beetle *Dendroides canadensis* are the most active AFPs known. Thirteen similar AFPs were purified and characterized. These consist of varying numbers of 12- and 13-mer repeating units with the consensus sequence Cys-Thr-X_3-Ser-X_5-X_6-Cys-X_8-X_9-Ala-X_{11}-Thr-X_{13} where X_3 and X_{11} tend toward charged residues, X_5 toward threonine or serine, X_6 toward asparagine or aspartate, X_9 toward asparagine or lysine, and X_{13} toward alanine. All of the cysteine residues are disulfide bridged, usually to the other cysteine within the repeat unit. This provides an extremely stable protein structure and probably positions the hydroxyl group of the highly conserved serine and threonine residues so they can hydrogen bond to ice, a requisite for the antifreeze activity. The secondary structure of these AFPs is ~46% ß-sleet, 39% turn, 2% helix and 13% random. Several low molecular mass salutes, mostly organic, were shown to enhance the activity of the AFPs several fold. The most active of these is citrate which enhances activity as much as sixfold. Succinate, malate, aspartate, glutamate, ammonium sulfate, glycerol, sorbitol, alanine and ammonium bicarbonate were also very effective. The mechanism of the enhancement is unknown.

14. SUBJECT TERMS

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Introduction: The overall goal of this project was to better understand the structure/function relationship of antifreeze proteins from the overwintering larvae of the beetle *Dendroides canadensis*. These are the most active antifreeze proteins that have been described. When the project was begun the complete sequence of one of these proteins was known, as was the partial sequence of three others.

1. **Molecular Characterization and Sequencing of *Dendroides canadensis* Antifreeze Proteins.** The deduced amino acid sequences of 13 different antifreeze proteins were determined from cDNA's and in some cases from peptide sequencing. The mature proteins consist of 12- and 13- mer repeat units with the consensus sequence consisting of Cys-Thr-X_3-Ser-X_5-X_6-Cys-X_8-X_9-Ala-X_{11}-Thr-X_{13} where X_3 and X_{11} tend toward charged residues, X_5 toward threonine or serine, X_6 toward asparagine or aspartate, X_9 toward asparagine or lysine and X_{13} toward alanine. The most interesting feature of these proteins is that throughout the length of the proteins every sixth residue is a cysteine. The sequence of DAFP-1, the most abundant of the *Dendroides* antifreeze proteins, is shown in Figure 1. The various DAFPs are quite similar to one another, the major difference being that of size, due to variation in the number of repeat units (7 to 11). However, while some DAFPs are very similar (DAFPs 1 and 2 differ at only two residues), others have much less sequence homology. Figure 2 provides the aligned sequence of all 13 DAFPs.

The initial peptide sequencing of the DAFPs was not productive because the N-terminus of the DAFPs was blocked, and the identification of the N-terminus was not possible from the cDNA's because of the presence of a signal peptide. Considerable effort demonstrated that the N-terminus is pyrogutamine.

The specifics of the DAFP sequence information and related information is contained in Duman *et al.* 1998 and Andorfer and Duman 2000.

2. **Disulfide Bridge Mapping**. As noted above, every sixth residence in the various DAFPs is a cysteine. These are completely conserved in all the 13 DAFPs. Therefore, it was important to

determine whether these cysteines are disulfide bridged, and if so which are involved. Figure 3 shows these linkages for DAFP-1. Note how the structure provided by the disulfide bridges position serine, and certain threonine, residues on one side of the protein (toward the bottom of Figure 3). The hydroxyl side chains of these residues are thus in position to bind to ice, a requisite for the antifreeze activity of the DAFPs. This disulfide bridge mapping information was published in Li *et al.*, 1998a.

Figure 4 gives the sequence of the various DAFPs, showing only those residues which are completely conserved in all 13 DAFPs. Thus, these residues are likely to be quite important for the function of these antifreeze proteins. This, and related information, is soon to be published in a review on insect antifreeze proteins (Duman, 2000).

3. Secondary structure of DAFPs. The secondary structure of the DAFPs was determined using infra-red and circular dichroism (CD) spectroscopies. The disulfide bridges impose significant constraints on potential secondary structural features (i.e., a number of three-residue γ-turns) which may lead to unusual infrared and CD spectra that require special interpretation. At 25 ° C the DAFPs contain ~46% β-sheet, 39% turn, 2% helix, and 13% random structure. In the presence of ice there is a slight increase in helix and β-sheet structures and a decrease in both turn and especially random structures. This change in the presence of ice may reflect a certain amount of flexibility in the DAFP structure. These structural changes may permit an improved lattice match between the DAFPs and ice, a requisite for the noncolligative freezing-point-depressing activity of the DAFPs (Li *et al.*, 1998b).

4. Enhancement of *Dendroides* antifreeze protein activity by solutes of low molecular mass. Generally, the magnitude of the antifreeze protein activity (also known as thermal hysteresis) depends on the specific activity and the concentration of the antifreeze protein. However, previous work (Wu and Duman, 1991) has shown that the activities of *D. canadensis* AFPs were enhanced by the presence of certain proteins. Work funded by this grant demonstrated that, in addition, several low-molecular-mass solutes enhance the thermal hysteresis activity of DAFPs. The most active of these is citrate, which increases the thermal hysteresis nearly sixfold from 1.2°

C in its absence to 6.8 ° C. Solutes which increase activity approximately fourfold are succinate, malate, asparate, glutamate and ammonium sulfate. Glycerol, sorbitol, alanine and ammonium bicarbonate increased thermal hysteresis approximately threefold. Interestingly, 0.5 M sodium sulfate eliminated activity. Solute concentrations between 0.25 and 1 M were generally required to elicit optimal thermal hysteresis activity.

Glycerol is the only one of these enhancing solutes that is known to be present at these concentrations in overwintering *D. canadensis,* and therefore the physiological significance of most of these enhancers is unknown. The mechanism(s) of this enhancement is also unknown (Li *et al.*, 1998c).

5. Expression of *D. canadensis* DAFPs. All of the above described work was done on natural proteins purified from *D. canadensis*. A primary objective of this study was to express the various cDNA clones of these AFPs to provide a more ready source of proteins for study. However, the initial attempts at expression using a yeast (Picchia) system were not successful. Large amounts of protein were produced, but it was not active, presumably because it was not folded properly. Recently an E. coli bacterial expression system (pET, Novagen) has been successfully used to express certain DAFPs (1, 2, 4, 8, 9, 10) in active form. However, although the other seven antifreeze proteins are produced in large amounts, they are not active.

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* Supported by this grant.

Graduate thesis supported by this grant.

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2. Cathy Ann Andorfer (1998) Seasonal variation in the expression of antifreeze proteins in *Dendroides canadensis* beetle larvae and isolation of novel antifreeze proteins. M. S. Thesis. University of Notre Dame.

Figure Legends

Figure 1. Sequence of *Dendroides* antifreeze protein -1 (DAFP-1) showing the seven 12- or 13-mer repeating units comprising the mature protein. Note that the amino terminus is pyroglutamine.

Figure 2. Sequences of the 13 DAFPs showing the repeating units.

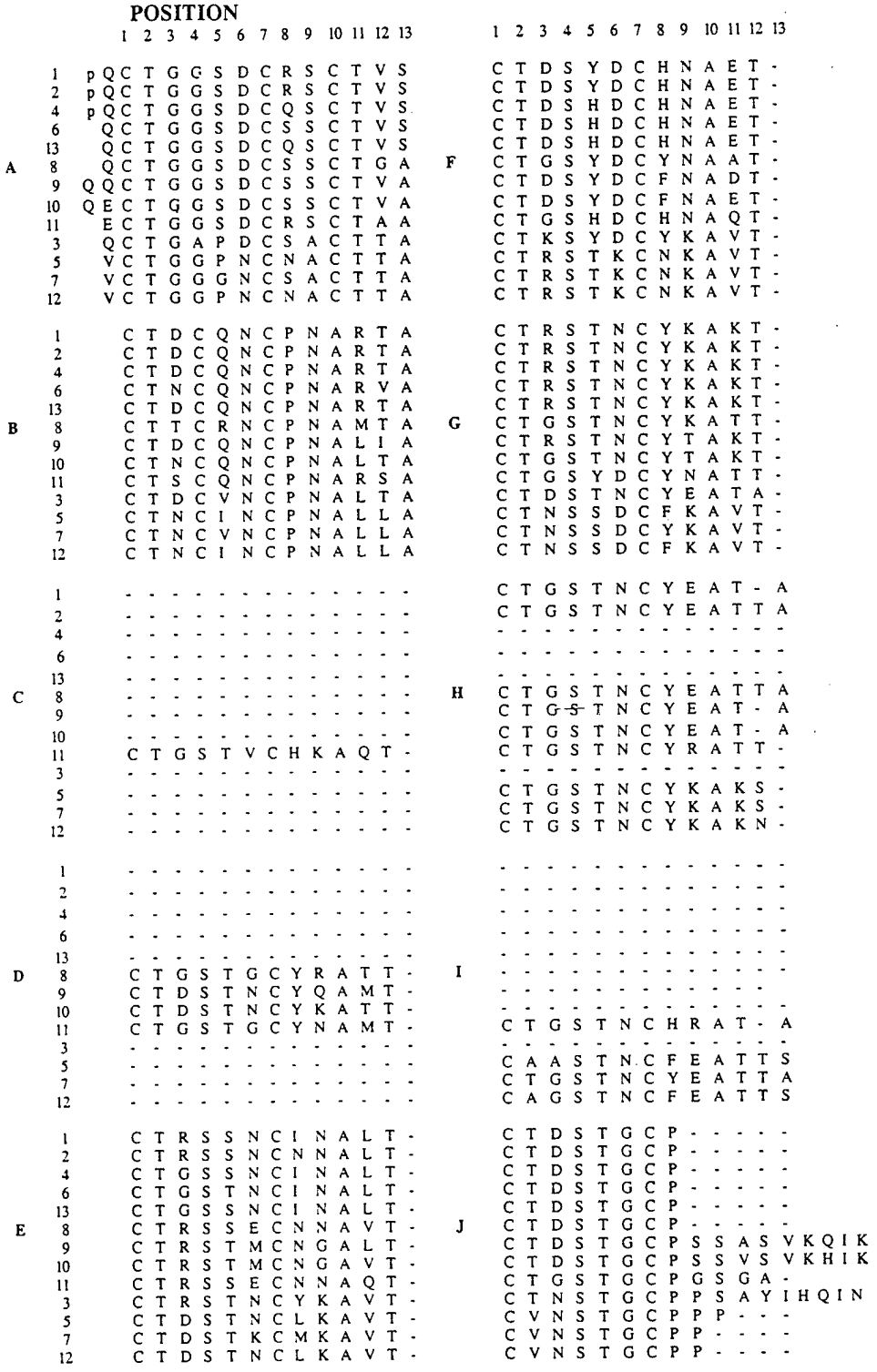
Figure 3. Sequence of DAFP-1 showing the locations of the disulfide bridges. Letters at the top designate the various repeats.

Figure 4. Consensus sequence of all 13 DAFPs showing the locations of disulfide bridges. Only positions having complete identity in all 13 DAFPs are indicated. Numbers designate the cysteine residues involved in disulfide bridges. Letters at the top indicate the various repeats. Length heterogeneity and c-termini beyond proline (where present) are not indicated.

DAFP-1

POSITION

REPEAT		1	2	3	4	5	6	7	8	9	10	11	12	13
A	pQ	C	T	G	G	S	D	C	R	S	C	T	V	S
B		C	T	D	C	Q	N	C	P	N	A	R	T	A
C		C	T	R	S	S	N	C	I	N	A	L	T	-
D		C	T	D	S	Y	D	C	H	N	A	E	T	-
E		C	T	R	S	T	N	C	Y	K	A	K	T	-
F		C	T	G	S	T	N	C	Y	E	A	T	A	-
G		C	T	D	S	T	G	C	P					


POSITION
1 2 3 4 5 6 7 8 9 10 11 12 13
A
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C
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E
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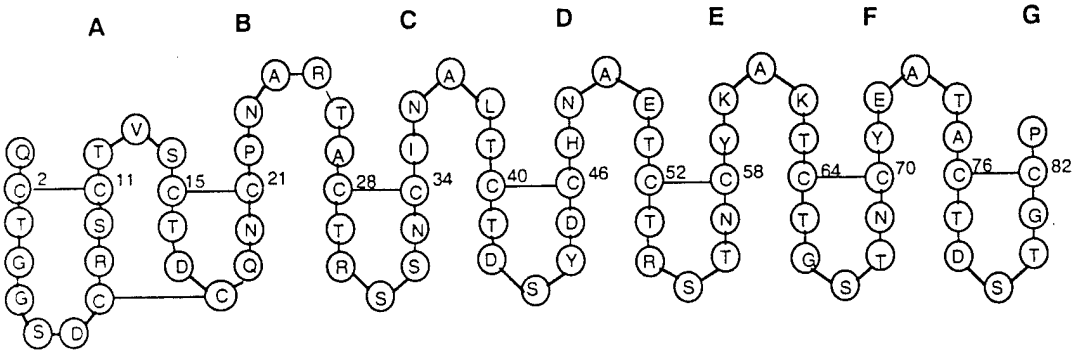
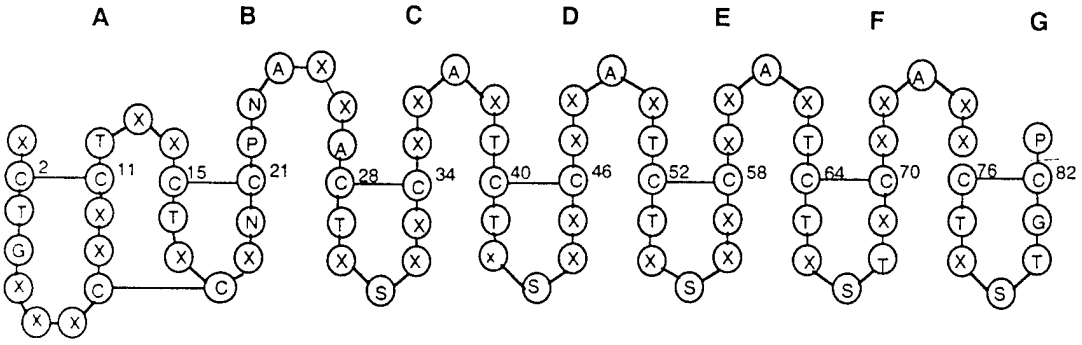
DAFP-1

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